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(54) Title: ATTENUATED VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS

(57) Abstract: The invention relate to the field of prevention or treatment Infectious Bursal Disease virus infections. The invention provides a method for the generation of attenuated vvIBDV and vaccines based thereon through alteration of the primary sequence of an essentially untranslated region of the viral nucleic acid.

Title: Attenuated very virulent Infectious Bursal Disease Virus

The invention relates to the field of prevention or treatment Infectious Bursal Disease virus infections.

Infectious Bursal Disease (IBD), an infectious disease among young chickens, was first recognized in 1957 in Gumboro, Delaware, USA. As a result, the disease is often referred to as Gumboro. Not long after IBD was first reported, it was being recognized in poultry populations throughout the world. IBD is caused by a virus (IBDV) classified as a Birnavirus. Two different IBDV serotypes exist: serotype I and II. Isolates belonging to serotype I are highly pathogenic for chickens. Serotype II isolates, which are mainly recovered from turkeys, have never been reported to induce clinical signs in chickens and are regarded as apathogenic.

IBDV infections were initially recognized by whitish or watery diarrhea, anorexia, depression, trembling, weakness, and death. This clinical IBD was generally seen in birds between three and eight weeks of age. The course of the disease runs approximately 10 day in a flock. Mortality usually ranges from 0-30 percent. Field reports suggest that leghorns are more susceptible to IBDV than broiler type chickens. Subclinical IBD was later recognized and is a greater problem in commercial poultry than the clinical disease. It is generally seen in birds less than three weeks of age. This early infection results in a depletion of lymphoid cells in the bursa of Fabricius. The bird is immunologically crippled and unable to respond fully to vaccinations or field infections. In susceptible chickens, damage caused by IBDV can be seen within two to three days after exposure to virulent virus. Initially, the bursa swells (3 days post-exposure) with edema and hemorrhages and then begins to show atrophy (7-10 days). IBD virus is especially cytopathic to certain B-lymphocytes. The highest concentration of these specific B-lymphocytes is found in the bursa. Destruction of the B-lymphocytes by IBD field virus may result in an incomplete seeding of these cells in secondary lymphoid tissue. As a result of the depletion of B-lymphocytes, surviving birds are immunocompromised during the remaining of their live time.

IBDV is found worldwide, and IBDV specific antibodies have even been found in Antarctic penguins. The prevalence of clinical IBD is relatively low compared to the prevalence of subclinical IBD. IBDV is very resistant to common disinfectants and has been found in lesser mealworms, mites, and mosquitoes. These facts correlate with field experience of reoccurring IBD problems on a farm, despite clean-up efforts. Infection with IBDV results in a strong antibody response, which is capable of neutralizing this virus. Most likely as a result of vaccination, antigenic variant isolates of serotype I were isolated in the Delaware area (USA). These isolates have been shown to cause bursa atrophy in as little as three days post-infection without inflammation of the bursa. Despite their change in antigenicity these antigenic variants do not form a distinct serotype. After the appearance of antigenic variant IBDV isolates in the USA, the poultry industry in European countries was hit by outbreaks of IBD caused by a very virulent serotype I IBDV (vvIBDV). These very virulent field isolates were capable of establishing themselves in the face of high levels of maternal antibodies which normally were protective. These vvIBDV cause more severe clinical signs during an outbreak and are now found globally (e.g. Europe, Japan, Israel and Asia).

IBDV belongs to the family of Birna viruses which include Infectious Bursal Disease Virus (IBDV) isolated from chickens, Infectious Pancreatic Necrosis Virus (IPNV) isolated from Fish, Drosophila X Virus (DXV) isolated from fruit fly, and Tellina virus (TV) and Oyster Virus (OV) both isolated from bivalve molluscs. Birna viruses have a dsRNA genome which is divided over two segments (the A- and B-segment). The A-segment (3.3 kbp) contains two partly overlapping open reading frames (ORFs). The first, smallest ORF encodes the non-structural Viral Protein 5 (VP5, 17 kDa). The second ORF encodes a polyprotein (1012 amino acids, 110 kDa), which is autocatalytically cleaved. From SDS-Page analysis of in vitro translated IBDV RNA it is known that the polyprotein is rapidly cleaved into three proteins: pVP2 (48 kDa), VP4 (29 kDa) and VP3 (33 kDa). During in vivo virus maturation pVP2 is processed into VP2 (38 kDa). VP2 and VP3 are the two proteins that constitute the single shell of the virion. The B-segment (2.9 kbp) contains one large ORF, encoding the 91 kDa VP1 protein. This protein contains a consensus RNA dependent RNA polymerase motive (Bruenn, 1991). Furthermore, this protein has been reported to be linked to the 5'-ends of the genomic RNA segments (Viral Protein genome-linked, VPg).

The nucleotide sequence of internal parts of a large number of IBDV isolates has been determined, and deposited in databases (GenBank). Furthermore Mundt and Muller 1 have determined the 5'- and 3'-termini of several IBDV isolates (CU-1, CU-1M, P-2 and 23/82). By combining the internal and terminal sequences, Mundt and Muller established the complete nucleotide sequence of the serotype I A-segment (3261 bp) and B-segment (2827 bp). Knowing the complete dsRNA sequence of IBDV genome, Mundt and Vakharia succeeded in producing infectious IBDV from the cDNA 2. The full length cDNA of IBDV, preceded by a T7 promoter, was thereby used as a template for T7 RNA polymerase using a method described by Weiland and Dreher 3. This in vitro generated mRNA, containing a cap-structure at its 5'-end, was subsequently transfected into eukaryotic cells (VERO cells) using a liposome formulation (Lipofectin, GibcoBRL). The supernatant of the transfected cells contained infectious IBDV after incubation during 36h in the CO_2 incubator at 37^{o}. Furthermore Boot et al., 4 have recently published a method for generating mosaic infectious vvIBDV, using a T7 RNA polymerase expressing helper virus (Fowlpox T7) and co-transfection of the plasmids containing the full length cDNA of the A- and B-segment. Although cDNA of IBDV can be used to produce infectious IBDV, the exact mechanism of replication has not been elucidated yet. Data exist which are in support of a semi-conservative genome replication model for Birnaviradae.

While the poultry industry in the USA was affected by antigenic variant strains of IBDV, the poultry industry in Europe was affected by very virulent IBDV (vvIBDV) isolates). The vvIBDV isolates cause more severe clinical signs during an outbreak and are able to break through levels of antibodies which are protective against classical IBDV isolates. The molecular determinants which distinguish vvIBDV from classical IBDV isolates are not exactly known. No specific antibodies, that exclusively recognize the vvIBDV isolates have been described yet. The lack of discriminating antibodies makes direct diagnosis difficult. Most attention has been given to sequence comparison between the hypervariable region of VP2 of classical isolates and of very virulent isolates. Sequence analysis of the vvIBDV isolate UK661 showed that only three unique (i.e. not found in non-vvIBDV isolates) amino acid substitution are present within the hypervariable region of the VP2 protein. One amino acid substitution is present within the remaining part of the pVP2 protein, while 5 unique amino acid mutations are present within the VP4 encoding part of the polyprotein and 6 in the VP3 encoding part. The smaller ORF of the UK661 isolate A-

segment, encoding the VP5 protein, contains 2 unique amino acid substitutions. Additionally 16 unique amino acid substitutions were found in the VP1 protein encoded by the B-segment of this vvIBDV isolate. The virulent phenotype of the vvIBDV might be influenced by each of these amino acid substitutions, and even (silent) nucleotide substitutions within the coding or non-coding parts of either the A- or B-segment may contribute to the altered phenotype of the vvIBDV isolates.

The goal of vaccination against IBD is prevention of subclinical and clinical IBD and the economic aspects of each. Effective vaccination for IBD can be divided into the following categories:

- Protection of the developing bursa in broilers, breeders and layers,
- Prevention of clinical disease in broilers, breeders and layers,
- Priming and boosting of breeders.

To minimize the immunosuppressive effects of IBDV, the young chick must be protected. Protection of the very young can be achieved through high enough levels of maternal antibodies passed from the breeder hen to her progeny. Vaccination of the very young chick itself may not be successful since onset of protection after vaccination is between three and five days. When a bird, lacking maternal antibodies against IBDV, is exposed to a pathogenic IBDV field strain, damage will occur within 24-48 hours.

Generally the early vaccinations of the breeders serve as priming. In most situations, this single vaccination is not considered to be adequate. Boosting is the term commonly associated with the administration of a final IBDV vaccination prior to the onset of lay. This is done to increase the level of circulating antibodies in the hen and hence the maternal antibodies in the progeny. Both inactivated (oil emulsion) and live IBDV vaccines have been used for this purpose. The use of a live vaccine in older birds will result in an increase in antibodies; however, large variations in antibody titers are often seen. These variations result in progeny becoming susceptible to field challenge from as early as a few days after hatching to 21 days after hatching. The use of inactivated IBDV vaccines gives a higher antibody titer as well as a decrease of variation between antibody titers of birds belonging to the same flock. The levels of maternal antibodies needed to neutralize IBD vary with the invasiveness and pathogenicity of the field strain. In practical terms, if a very virulent IBDV isolate is present, higher maternal antibody levels are desired. Yet, for effective vaccination, avoiding interference with maternal antibodies is needed to prevent clinical IBD.

Clinical IBD is typically seen between three and six weeks of age. The immune response of the chick must be stimulated as the passive protection is declining. The timing of the active vaccination may be estimated by the breeder or chick titer and the half-life of antibodies of approximately 3.5 days.

The levels of maternal antibodies tend to vary within a population. This variation might be a result of variation in the antibody levels of the breeder hens. Also the mixing of progeny from several breeder flocks (e.g. combination of breeders of different age; breeders vaccinated with live vaccine and those with oil emulsion vaccine) results in variation of IBDV antibodies between chicken belonging to the same flock. If the coefficient of variation (CV) in mean maternal antibody titers is too wide, it may be recommended to vaccinate twice (with a 10-day interval) or to vaccinate early with a hot vaccine (in the presence of a high antigenic pressure).

The average titer of antibody against IBDV in a flock will decline in time. As a result of the decrease in average antibody titers, an immunity gap will occur. The best results are obtained if the immunity gap is as short as possible and is as early as possible, with a minimum of 2 weeks after hatching. There should be at least sufficient immunity after active vaccination at the age of 4 weeks, since many handlings occur in the houses from that time point on with risks of introducing field virus. Therefore, farmers like to vaccinate at 2 weeks or even before. Intermediate vaccines are unable to break through the average IBDV antibody titer of the broiler at two weeks after hatching. If there is a high variation in mean maternal antibody titers, some chicks will be effectively vaccinated with intermediate vaccines, others not. To circumvent those problems, hot vaccines are being used. A drawback of usage of hot vaccines is that the bursa of chickens with low to moderate maternal antibody titers will be (partly) damaged.

There are a variety of IBDV vaccines available. Important aspects in vaccination strategies are the ability of the virus to replicate in the face of maternal antibody (invasiveness of the vaccine) and the spectrum of antigenic content (including antigenic variants). The ability of a vaccine virus to replicate in the face of maternal antibodies allows live vaccines to be categorized into three main groups: mild, intermediate, and intermediate plus or hot vaccines.

The initial vaccines for IBD were derived from classical IBDV isolates. These vaccines were moderately pathogenic IBDV strains with low passage numbers in embryonated eggs. When given to young birds with moderate or low levels of

maternal antibodies, these vaccines can cause extensive bursal atrophy resulting in immunosuppression. Mild vaccines were subsequently developed to be used in these young birds. The attenuation of classical IBDV was done in tissue culture systems. These vaccines are not immunosuppressive even when used in birds having no maternal antibodies. However, moderate and high levels of antibodies easily neutralize them. As breeder programs developed (including the use of adjuvant, inactivated vaccines), higher levels of maternal antibodies were generated in progeny. This reduces the effectiveness of these mild vaccines.

Intermediate strength vaccines were to overcome the inadequacies of the mild vaccines. Some of the intermediate vaccines are developed by cloning a field isolate on chicken cell cultures. Intermediate strength vaccines are capable of establishing immunity in birds with moderate levels of maternal antibodies. These vaccines will cause some bursal atrophy in birds without maternal antibodies, but are not immunosuppressive.

Hot and intermediate plus vaccines were developed after the first outbreaks with vvIBDV. These vvIBDV isolates could break through higher levels of maternal immunity than the vaccines that were on the market at that time. Vaccination with intermediate vaccines came always too late in situations with high infection pressure with vvIBDV. Hot vaccines consist of vvIBDV strains with low to moderate passage in embryonated eggs or bursa derived IBDV of chickens infected with vvIBDV isolates. They are able to circumvent maternal immunity at an earlier age than intermediate vaccines and spread more within a flock. If intermediate plus and hot vaccines are used in chickens with moderate to high levels of maternal antibodies, there is no negative side effect on the bursa. If these vaccines are used in chickens with low to moderate levels of maternal immunity, this causes depletion of lymphoid cells in the bursa and a severe depletion of peripheral blood-B cells. Although a quick recovery of bursal function has been observed, these vaccines should be used with precautions.

Live vaccines must be given in a way in which the virus will reach the bursa where it will multiply and induce an immune response. Possible routes for application of live vaccines include drinking water, spray, subcutaneous and in ovo. Inactivated IBD vaccines are used in broiler breeders. They differ in some of the same ways as live vaccines. Their efficacy depends upon the spectrum of antigens they

contain. Injectable oil-emulsion products may be given subcutaneously or intramuscularly.

The invention provides a method for the generation of replicative, albeit preferably attenuated Birna virus, preferably suitable of replication in cell-culture, preferably IBDV such as vvIBDV and vaccines based thereon through alteration of the primary sequence of an untranslated region of the viral nucleic acid. The 3'terminal, untranslated nucleotides o the coding RNA strands of the IBDV A- and B-segment are predicted to fold into a stem-loop structure (Fig. 1 and 2). There was neither earlier direct biochemical prove that this predicted stem-loop structure indeed exist nor were data available indicating that these stem-loop terminal nucleotides play a role in viral replication. For example, Nagarajan et a (Can. J. Vet. Res. 65:89-96, 2001), while finding nucleotide changes in the 3' non-coding regions of segments A and B of IBDV, fail to identify such stem-loop structures, or even provide a full sequence from which such stem-loop structure may be deduced. Furthermore Boot et al (Virology 265: while evaluating the effect of the length of the 3'terminus of the A segment plus strand of IBDV, suggest that during reproduction, missing nucleotides are likely restored using complementary bases of a stem-loop structure as a template, but fail to recognize that providing alternative stem-loop structure (i.e. not being restored to wild type, but bearing mutations that maintain a stem-loop structure as described herein) contributes to desired attenuation. Clearly, Weber et al (JGV 82:805-812, 2001) studying 5 alterations of the non-coding region of the A-segment infectious pancreatic necrosis virus plasmids that lead not to viable virus, does not recognize the importance of such a stem-loop structure.

Having acquired the insight that stem-loop terminal nucleotides play a role in viral replication, the invention now provides a method for the preparation of an attenuated Birn virus comprising providing an alteration of the primary sequence of a 3'untranslated regio of its genomic RNA. In particular, the invention provides a method wherein said alteratio comprises a nucleotide substitution or deletion. To advantagously attenuate, it is preferred that said alteration provides a secondary stem-loop structure of said region with a predicte stability within a range of from -8 kcal/mol to -15 kcal/mol, most preferred stability is foun in the range -10 kcal/mol to -13 kcal/mol, bringing it again close to wild-type stability. Mos preferred is when the resulting predicted stem-loop structure has essentially the same forr and build as its wild-type counterpart, having a predicted stability within the range -11 kcal/mol to -12 kcal/mol.

The invention also provides virus obtainable by a method according to the inventio which advantageously can be used in a vaccine as provided herein. Changing the predicted stem-loop, as provided here, affects replication, and makes the virus suitable for in vitro culture, thereby resulting in easier culture techniques that allow the accretion of more relevant viral antigenic mass for incorporation in a killed vaccine.

However, in a preferred embodiment, the invention provides a vaccine wherein said virus live. The fact that substitutions in the 5'- or 3'-UTR of the A or B-segments results in an attenuated phenotype is a significant new finding which especially provides a new generation of attenuated very virulent IBDV live vaccines. A further possible application i the production of a reassorted serotype I IBDV, i.e. consisting of the A-segment of a wild-type vvIBDV (for example strain D6948) in combination with a 3'-UTR mutant B-segment of a cell culture adapted IBDV (for example strain CEF94). Another good candidate for the 3'-UTR mutated B-segment is indicated by having the sequence as found in the 4th passag originating from the pMB-1 to 10 plasmids (see Table 3). Especially those sequences whic result in a attenuated phenotype in vitro (i.e. the sequences as found in the fourth passage after co-transfection of pMB1, and -6) are likely candidates, and are examples of a Birna virus provided with an alteration of the primary sequence of an essentially untranslated region of its genomic RNA. Preferred is a virus wherein said alteration comprises a nucleotide substitution or deletion as for example shown in figure 3, but insertions can als be contemplated. Substitions or deletions essentially corresponding to those made in the infectious bursal disease virus as provided in the detailed description can easily be made f other Birna viruses, whereby, as said, it is preferred that for the 3'-UTR the shape essentially resembles wild-type stem-loop structure.

The invention also provides a reassorted virus according to the invention provided with an alteration of the primary sequence of an essentially untranslated region of its segment B of its genomic RNA whereby an essentially untranslated region of its segment A comprises a sequence characteristic for wild-type virus, or, vice versa, provided with an alteration of the primary sequence of an essentially untranslated region of its segment A o its genomic RNA whereby an essentially untranslated region of its segment B comprises a sequence characteristic for wild-type virus. It is for example herein provided that reassortments are done with a virus according to the invention wherein the mutated regio is from segment B, whereby for example the rescued viruses containing the vvIBDV A-segment and a mutated B-segment have the phenotype of an attenuated vvIBDV live vaccine, however, the reverse reassortments also result in the desired viruses for the

production of a vaccine, such as a poultry vaccine for the treatment or prevention of IBDV infections, be it a killed or modified live vaccine. In particular attenuated live vaccines are useful globally as a life vaccine against outbreaks of vvIBDV.

Detailed description

Material and Methods

Introduction of nucleotide deletions and substitutions in the cDNA plasmids of IBDV

A deletion of 12 nucleotides (nt 85 untill 97 nt; GenBank accession AF240686) was generated using a fusion PCR approach and pHB-60 as template and mutant primers VP5-D23P (CTCCTCCTTC TACAATGGTT AGTAGAGATC) and VP5-D23M (CTCTACTAAC CATTGTAAG GAGGAG). Furthermore two nucleotides were changed in comparison to the wild-type A-segment sequence (GenBank accession number AF240686). The first substitution was introduced at position nt 889 where we changed adenine into cytosine, and the second substitution was at position 980 where we changed guanine into adenine. These two mutations results into a cell culture adapted virus after rescue, as the amino acids at position 253 and 284 were changed into histidine and threonine, respectively, in comparison to the wild-type amino acid sequence of very virulent IBDV. The nucleotide substitutions were introduced by using the fusion PCR approach (see above), yielding plasmid pJH60-HT-D23.

Mutations in the 3'-UTR of the B-segment cDNA plasmid of CEF94 (pHB-34Z) were introduced by using oligonucleotides which contain one or more nucleotide substitutions compared to the wild-type B-segment stem-loop sequence, see Table 2). A polymerase chain reaction was performed using a mutant oligo's in combination with oligo BC10 (5'-gctctagaTCAAGAACCCACAGACCG-3'), pHB-52 as template, and Pwo polymerase (Boehringer Mannheim) as enzyme. The pHB-52 plasmid is a derivative of pHB-34Z that contains a NgoMIV restriction site at nt 2741. This PCR fragment was subsequently purified, digested with NgoMIV and used to replace the corresponding part of plasmid pHB-52 (digested with NgoMIV and SmaI). Cloning

was performed using the Rapid DNA-ligation kit of Boerhingher Mannheim (in accordance with the suppliers instruction). Mutant plasmids pMB-1 through pMB-13 were recovered from transformed E. coli (DH5alfa) cells using the Qiagen Tip-100 purification method according to the suppliers (Qiagen GmbH, Germany) instruction. The presence of the substitutions in pMB-1 through -13 was confirmed by sequence analysis. Secondary structure prediction of the altered 3'-UTR region of the coding-strand B-segment RNA was performed using the Mfold program (version 3.0).

Co-transfection of Fowlpox T7 infected QM5 cells with the IBDV cDNA plasmids

Purified plasmid pJH60-HT-D23 was co-transfected with plasmid pHB-43Z into QM5 cells. Purified pMB plasmids were co-transfected with the pHB-36W plasmid into QM5 cells. Plasmid pHB-34Z contains the B-segment cDNA of CEF94 preceded by a T7 polymerase promoter, and followed by the Hepatitis Delta Virus ribozyme. Plasmid pHB-36W contains the A-segment cDNA of CEF94 preceded by a T7 polymerase promoter, and followed by the Hepatitis Delta Virus ribozyme. Prior to co-transfection, the QM5 cells were infected for 1 hour with Fowl-Pox T7 virus. After transfection the cells were rinsed with PBS, covered with complete medium and incubated for 24h at 37^{o} C (5.0% CO_2). Subsequently, the cells and supernatant were freeze-thawed once, filtered through a 200-nm-pore-size filter (Acrodisc, Gelman Sciences) and stored at –20°C. All transfection experiments were performed 4 times independently. The amount of infectious rIBDV particles was determined by a $TCID_{50}$ (50% Tissue Culture Infectious Dose) assay. This analysis was performed by infecting fresh QM5 cells in a 96-wells plate with 10-fold dilutions of the filtered transfection lysates. rIBDV infected wells were visualized in an Immuno Peroxidase Monolayer Assay (IPMA) using an IBDV specific Mab directed against VP3.

Induction of CPE by rD6948^{HT}-D23-BC.

To assess to induction of cytopathic effect by rD6948^{HT}-D23-BC, we infected a fresh monolayer of QM5 cells with the supernatant of co-transfection experiments (see above). The CPE was in the monolayer was determined each 24h and was scored as – is no CPE; + is minor CPE; ++ is moderate CPE; +++ is complete CPE.

Serial passages of mutant rCEF94: To determine the stability of the introduced mutations we serially passaged the mutant rCEF94 for 4 times on QM5 cells. The mutant rCEF94 which appeared to have a non-wild-type B-segment 3'-UTR in the 4th passage were further passaged until the 10th passage. During each passage the freeze-thawed lysate of a culture was filtered through a 200-nm-pore-size filter (Acrodisc, Gelman Sciences), and 10% of the volume was used to re-infect a fresh culture.

Sequence determination of the B-segment 3'-UTR of rIBDV after serial passages: To determine the 3' termini of the plus strands of the B-segments of the rCEF94-MB1 to –MB9 of either the 4th or 10th passage, we purified genomic RNA of QM5 infected cells by differential centrifugation and Proteinase K treatment. Genomic dsRNA was disolved in 10 µl polyA buffer (50 mM Tris-HCL (pH 7.9), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 250 mM NaCl, 1 mM DTT, 50 µg/ml BSA, 0.04 units RNasin (Promega), in DEPC treated water) with 0.25 mM ATP, and 1 unit polyA polymerase (Pharmacia). After incubation at 37°C for 30 min the enzyme was denaturated by incubation at 75°C for 10 min. The polyadenylated dsRNA was purified by phenol/chloroform extraction (two times) and the purified RNA was precipitated by ethanol/NaAc. The dsRNA was dissolved in 25 µl DEPC-treated water. For the generation of single stranded copy DNA (cDNA) 10 µl of purified polyadenylated RNA was used and 4 µl AmpliTaq buffer II (10x, Perkin-Elmer), 4 µl $MgCl_2$ (25 mM, Perkin-Elmer), 4 µl DTT (0.1 M, Gibco), 2 µl dNTPs (10 mM each) and 4 µl UAPdT (5'-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3') primer (2 pmol/µl) were added. After incubation at 70°C for 2 min this mixture was immediately transferred onto ice. The dsRNA-primer mixture was subsequently incubated at 52°C for 2 min, after which 1 µl of reverse transcriptase (Superscript II, Gibco/BRL) was added, or no reverse transcriptase (negative control) and incubation at 52°C was continued for 1 h. The reverse transcription reaction was terminated by incubation at 75°C for 10 min. The genomic dsRNA and the RNA of the RNA/DNA hybrid were destroyed by addition of 0.5 µl mixture of RNase A (20 mg/ml, Calbiochem) and 0.5 µl RNase H (1.2 U/µl, Pharmacia), followed by incubation at 37°C for 20 min. To amplify the single stranded cDNA we used a nested PCR. For the first PCR we used the primers UAP (5'-GGCCACGCGTCGACTAG-3') and BC9 (5'-

gctctagaACTtGTGGAAACAAGCGA-3'), for the nested PCR we used the primers UAPN (5'-CCACGCGTCGACTAGTAC-3') and BC10 (5'-gctctagaTCAAGAACCCACAGACCG-3'). The following conditions were performed for the first PCR: 2 µl cDNA, 10 pmol of each specific primer, 2.5 mM $MgCl_2$, 1*AmpliTaq buffer II (Perkin-Elmer), 50 mM each dNTP and 1 unit of AmpliTaq polymerase (Perkin-Elmer), in a total volume of 50 µl. After incubation at 94°C for 1 min, the amplification was performed in 30 cycles through the different temperature levels: 94°C for 15 s, 52°C for 15 s, and 72°C for 60 s. For the nested PCR, 15 µl of the first PCR amplification reaction was separated in an agarose gel (1.5%) and the specific product (372 bp) was stinged 3 times with a needle and mixed in the 50 µl complete nested-PCR mixture. The conditions of the nested PCR were the same as in the first PCR with the following adaptations: a hybridization temperature of 54°C and 3.5 mM $MgCl_2$. The resulting nested PCR product (309 bp) was gel purified and sequence analysis was directly performed on the PCR products.

Growth Curve's of rIBDV of the 4th passage: To compare the replication ability of the mutant rCEF94 viruses of the fourth passage with the unmodified serotype rCEF94, we determined growth curves of all rCFE94 viruses which had a non-wild type B-segment 3'-UTR. QM5 cells were grown overnight (16 h) in a 60 mm cell culture dish. The medium was subsequently removed and 1 ml PBS containing rIBDV (Log_{10} $TCID_{50}$ between 6 and 7) was used to cover the cells. After one hour the supernatant was removed, the cells were rinsed four times with 8 ml of PBS, and 5 ml complete medium was added. At different time points (1h, 5h, 10h, 15h, 25h, and 50h post infection) samples were taken from the supernatant and stored at –20° C. The viral titer in each sample was determined in an $TCID_{50}$ assay (see above).

Results

To assess whether we could use modified IBDV as a live vaccine, we have deleted 12 nt of the 5'-non-coding region of the A-segment of vvIBDV corresponding to the sequence ACGCUAUCAUUG in CEF94 (see underlined sequence in Fig. 5). This fragment containing said deletion was introduced into a cDNA plasmid containing the full-length A-segment cDNA of D6948 vvIBDV, in which two additional nucleotide mutations were introduced at position 889 and 980 (see Material and

Methods). The two nucleotide mutations at position 889 and 980 result into a cell culture adapted phenotype of the rescued IBDV, as a histidine is present at position 253 and a threonine is present at position 284 of VP2. Rescue of IBDV from deletion plasmid (pJH60-HT-D23) was severely reduced, as the efficiency of rescued was approximately 100-fold reduced in comparison to virus from the cDNA plasmid which does not contain this deletion (pJH60-HT). Furthermore, we found that the appearance of CPE was severely reduced when a fresh monolayer of QM5 cells was infected with rD6948^{HT}-D23-BC in comparison to the corresponding virus (rD6948^{HT}-BC) which lacks this 12 nt deletion. Both these experiments show that the generated virus rD6948^{HT}-D23-BC is attenuated in comparison to wild-type IBDV.

Computer analysis of the secondary structure of the positive strand of the RNA genome segments A and B of IBDV predict that non-coding nucleotides present at the 3'-terminus of the plus-strand RNA will fold in a stem-loop structure. The shape of this stem-loop is unclear and might consist of two stem structures 5 or only one 6 (Fig. 2), depending on the number of nucleotides used for the secondary structure prediction. No direct biochemical data have been published supporting the presence of the predicted 3'-UTR stem-loop structure. Furthermore, if a stem-loop structure is indeed present during an infection in the natural host, no data exist that indicate or prove that this structure plays a role in replication and/or virulence of Birnaviruses. Solely based upon the computer prediction of the stem-loop structure a function for this potential stem-loop structure in protection against exonucleases 6, in transfer of ribosomal factors 6 in determining host-cell tropism 5, and in initiation of minus strand synthesis 6 has been suggested.

To investigate the presence and function of the predicted 3'-UTR stem-loop structure of the B-segment of IBDV we substituted specific nucleotides in the corresponding region of the B-segment cDNA plasmid (see Fig. 3). The resulting mutant B-segment cDNA plasmids (pMB-1 –13) were subsequently co-transfected with a wild-type A-segment cDNA plasmid (pHB-36W) into QM5 cells which had been infected with a recombinant Fowlpox virus containing the actively transcribed RNA polymerase gene of phage T7. The QM5 cells were incubated for 24h at 37°C (5% CO_2) after the co-transfection to allow the initially generated rIBDV to amplify. The amount of infectious IBDV particles ($TCID_{50}$) after this incubation period was determined for all mutants and also for QM5 cells which had been co-transfected with the unmodified A-and B-segment cDNA plasmids of the CEF94 IBDV strain (see

Table 2). The substituted nucleotides have apparently an influence on the rescue efficiency and/or replication competence of IBDV, as transfection with modified B-segments cDNA plasmids resulted in all cases in a decreased amount of infectious viral particles at 24h after transfection, in comparison with the rescue of unmodified rCEF94, which was performed in parallel (Table 2). Although we do not know the reason for the reduced rescue efficiency at this stage, it is clear that the introduced substitutions must have a direct effect on the replication of IBDV, because they are all are located in an untranslated region (the 3'-UTR), which excludes an indirect effect through substitutions in encoded viral proteins.

Although all introduced mutations had a negative effect on the rescue efficiency, we found the most dramatic effect (no viable virus) when nucleotide substitutions were present at the base of the stem-structure (Table 2; pMB-11, -12 and –13). The increase in the length of the predicted stem region of rIBDV–12, and –13, which is accompanied by an increase in the stability of the predicted stem-loop structure (Fig. 3), is in our opinion the cause of the non-viability of the corresponding virus. The fact that pMB-11 does not yield a viable virus results probably from the disruption of the non-baseparing cytosine tract which is always present at the extreme 3'-UTR of Birnaviruses. It has been suggested that these free cytosines can basepair with one or more guanines with are covalently linked to the VPg (protein primed innitiation of the minus strand synthesis) 7. The length and or the stability of the 3'-UTR predicted stem-loop structure is apparently of major importance for viral replication.

Most of the viable mutant rIBDV's (rCEF94-MB1 through –MB9) were passaged 4 times and used to determine the nucleotide sequence of the 3'-UTR of the B-segment (Table 3). This analysis showed that substitutions had occurred in all of the rCEF94 viruses in comparison with the corresponding cDNA plasmid, and that these substitutions were all present in the region of the predicted stem-loop structure. Some of these mutations (found for example in the 4th passage of rCEF94-MB1, -MB2, -MB4 and -MB6, Table 2) are so-called second site mutations, leading to mutant viruses which have both the cDNA encoded substitutions and substitutions at another position in the predicted stem-loop structure. The sequence of some other mutant rCEF94 strains of the third passage (i.e. rCEF94-MB7 and –MB8, Table 3) showed substitutions which restored the wild-type sequence (reversion), while yet

other viruses (rCEF94-MB3, -MB5 and -MB9, Table 3) appeared to have a combination of substitutions at first and second sites. Virus resulting from the 4th passage after co-transfection of the wild-type A-segment with pMB-4 and pMB-5 appeared to be a mixed culture (see Table 3), where two different nucleotides were found at the same position in the 3'-UTR of the B-segment of the purified virus. These viruses cultures consist apparently of mixed populations, both originating from the same parental cDNA sequence.

Rescued mutant CEF94 from the fourth passage was not only used to determine the sequence of the 3'-UTR of the B-segment, but also used to determine the replication kinetics on QM5 cells (growth curve). For mutant rCEF94 which had acquired second site mutations, we found in some cases (rCEF94-MB1, and –MB6) a clear reduction in the replication kinetics in comparison with rCEF94 (Fig. 4), as both the titer at 25h and the final titer at 50h were considerably below the values for unmodified rCEF94. Whether rCEF94-MB9 also has a reduced replication is not clear yet, as the final titer at 50h equals 8.0 (like unmodified rCEF94), while the titer at 25h hour is ten-fold below the titer of unmodified rCEF94 (7.0 versus 8.0, Fig. 4). For the other rCEF94 mutants which acquired second-site mutations (i.e. rCEF94-MB2, -MB3, -MB4, and –MB5) we found replication kinetics equalling unmodified rCFE94 (Fig. 4).

To determine whether the introduced substitutions and acquired second-site substitutions were stable during prolonged cultivation we serial passaged all rCEF94-MB1 through -MB6, and –MB9 viruses of the 4th passage. Nucleotide sequence determination of the 3'-UTR of the plus-strand of the B-segment of purified virus of the 10th passage showed no differences compared to the nucleotide sequences as found in the 4th passage. The sequence of the stem-loop structure of recombinant virus which had acquired second-site mutations at an early stage (before the 4th passage) is apparently maintained during prolonged cultivation (at least 10 passages), even when the mutant virus has reduced replication kinetics, as is the case for rCEF94-MB1 and –MB6. Two of the rescued viruses (rCEF84-MB4 and –MB5) appeared to consist of a mixed population in the 4th passage. Although the relative amount of the two different sequences differed in the 10th passage in comparison with the 4th passage, both sequences (as represented in Table 3) are also present in the 10th passage. This indicates that the relative replication rates of viruses containing one or the other sequence do not differ significantly.

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Further example

A modified stem-loop 3'-UTR stem-loop in the B-segment of Infectious Bursal Disease Virus is maintained during replication in cell culture and chickens

Introduction

Infectious Bursal Disease Virus, an avibirnavirus, is the causative agent of the highly contagious disease among chickens known as Gumboro disease (Cosgrove, 1962). The genome of birnaviruses consists of two segments of double stranded RNA (dsRNA). The largest IBDV dsRNA segment (A-segment, 3260 bp) contains two partly overlapping open reading frames (ORFs). The first, smallest ORF encodes the non-structural, and non-essential Viral Protein 5 (VP5, 145-149 amino acids, 17 kDa). The second ORF encodes a polyprotein (1012 amino acids, 110 kDa), that is autocatalytically cleaved to yield the viral proteins pVP2 (48 kDa), VP4 (29 kDa) and VP3 (33 kDa). During *in vivo* virus maturation pVP2 is processed into VP2 (41-38 kDa), by the viral protease (VP4). The smaller IBDV B-segment (2827 bp) contains one large ORF, encoding VP1 (877-881 amino acids, 91 kDa). VP1 is the RNA dependent RNA polymerase, and is present both in its free form and covalently linked to the 5'-ends of the genomic RNA segments (Viral Protein genome-linked, VPg) (Dobos, 1993).

Only limited data exist initiation of translation and replication of birnaviridiea. The presence of the extraordinairy large VPg at the 5'-end of the positive strands of the two genomic segments of the birnaviruses has been shown (Calvert *et al.*, 1991; Muller and Nitschke, 1987). All 3'-terminal sequences (positive and negative strand of both the A-and B-segments) of IBDV and IPNV are ending with at least two constitutive cytosines, supporting the expectation that the 5'-termini of the non-coding strands also will have a covalently linked VPg. These 3'-UTR cytosines are probably essential to allow protein-primed initiation of transcription of the RNA using the VPg linked guanines (Boot et al., 1999). For poliovirus it has recently been described that a cis-acting replication element (cre) stem-loop structure, located in the coding region of 2C, is necessary for the 5' phosphodiester linkage of two 2

constitutive uracils to the small VPg. Whether such a cis-acting element is also involved in first steps of the VPg formation, the 5' phosphodiester linkage of an unidentified serine of the VP1 to 1 or 2 guanines, is yet unknown. The messenger form of positive stranded birnavirus RNA is likely to contain also this large VPg, where it likely functions in the initiation of translation of the mRNA, as birnaviruses do neither have a 5'-cap structure nor an internal ribosoom bindings site (IRES) to recruit host cell encoded the pre-initiation factors.

The 3'-end of the birnavirus mRNA differs from most cellular mRNA, as no poly-A tail is present. Poly-A tails have been shown to function in the protection against 3'-5'exonucleases degradation. Furthermore, an poly-A tail enhances the translation efficiency via the interaction with the poly-A binding protein (PABP), which circularizes the mRNA through its interaction with eIF4G, the scaffolding component of the pre-initiation complex (Gallie, 1998). The competition for initiation of translation between viral and messenger mRNA is an important issue, and different viruses are using different strategies to out-compete cellular mRNA's for the translation initiation factors. How the non-poly-A IBDV mRNA resolves the problems of recruiting translation factors without a poly-A tail is unclear at the moment. For rotavirus, a segmented dsRNA virus with capped, non-poly-A mRNA's, it has been shown that a viral protein (NSP3) mimics the function of PABP, by binding both the 3'-UTR's of the viral mRNA's and with the eIF4G ((Piron et al., 1998, Poncet et al., 1994).

Folding of the IBDV 3'-UTR regions in a particular 3-D structure likely functions in protection against degradation, enhancement of translation initiation, and initiation of replication. Secondary structure prediction of the 3-UTR region of both he A- and B-segment of IBDV showed that a distinct small stem-loop structure can be formed by the last 25 nucleotides of the 3'-UTR (Boot et al., 1999). To determine whether introduced mutations are tolerated, and/or lead to compensating or reversion mutations we have changes several nucleotides predicted to be involved in the formation of the stem-structure of the B-segment stem-loop. Rescue of infectious virus from these cDNA plasmids containing these mutations was in all cases impaired. However, after one passages on a cell line, the replication kinetics of these viruses appeared to be restored, as the single step growth curves were

indistinguishable from wild-type virus . Sequence analysis of the 3'-UTR of serial passaged virus originating from the mutagenized stem-loop cDNA plasmids revealed that in most cases additional mutations had been acquired, which of compensated the artificial introduced mutations. An rescued virus containing a modified stem-loop structure that contains four nucleotide substitutions, but preserving the overall predicted secondary structure, was rescued, and in vitro indistinguishable from wild-type virus. Sequence analysis showed that the modified stem-loop structure was fully preserved after 4 serial passages. The virulence of a segment reassorted very virulent IBDV, consisting of a wild-type A-segment and an B-segment containing this modified stem-loop structure appeared to be roughly equal to that of a virus containing an unmodified stem-loop structure, as the damage of the bursa of Fabricius yielded an equal score (> 4) at 3 weeks post infection. Based upon our mutagenesis studies we concluded that it is indeed possible to alter primary sequence of the 3'-UTR (as long as the secondary structure is not altered) without a dramatic effect on the replication capabilities. Using this knowledge it will be possible to design modified 3'-UTR stem-loop structures which are maintained during in vitro and in vitro replication, but which shows a reduced virulence. Strains with modified 3'-UTR stem-loop mutations and a reduced virulence be used as IBDV live vaccine.

Material and Methods

Viruses, cells, eggs and antibodies. The classical IBDV isolate CEF94 is a derivative of PV1, which is able to replicate in non-B-lymphoid cells (Boot et al., 1999). The D6948 strain is a very virulent field isolate (Poultry Health Service, Doorn, The Netherlands, 1989), which only grows in primary B-lymphoid cells (Boot et al., 2000). Recombinant fowlpox T7 virus containing the T7 polymerase gene (Britton et al., 1996) was a kind gift of M. Skinner (Compton Laboratory, Berks, United Kingdom). QM5 cells (Antin & Ordahl, 1991) were maintained by using QT35 medium (Gibco-BRL), supplemented with 5% Fetal Calf Serum (FCS) and 2% antibiotic solution (Boot et al., 2000) (compleet medium). Embryonated SPF eggs (XXX) were obtained from Charles River Laboratories, and incubated for 11-days before infection with rIBDV containing solution (200 µl) using the CAM route. The group specific monoclonal antibodies 9.7 which recognizes VP3 of IBDV was prepared in our laboratory by using purified CEF94 as an antigen.

Introduction of nucleotide substitutions in the cDNA plasmid of the B-segment.

Mutations in the 3'-UTR of the B-segment cDNA plasmid of CEF94 (pHB-34Z, (Boot et al., 1999)) were introduced by using specific oligonucleotides (MB-1 through MB-13, and MB-1R2R), which constituting the 5'-UTR sequence of the negative B-segment strand, and one or more nucleotide substitutions compared to the wild-type CEF94 sequence (see Table 5). A polymerase chain reaction were performed using one of the mutant oligo's in combination with oligo BC10, pHB-52 as template, and *Pwo* polymerase (Boehringer Mannheim) as enzyme. The pHB-52 plasmid is a derivative of pHB-34Z that contains a *Ngo*MIV restriction site at nt 2741. These PCR fragments were subsequently purified, digested with *Ngo*MIV and used to replace the corresponding part of plasmid pHB-52 (digested with *Ngo*MIV and *Sma*I). Cloning was performed using the Rapid DNA-ligation kit of Boerhingher Mannheim (in accordance with the suppliers instruction). Mutant plasmids pMB-1 through pMB-13 were recovered after transformation and selection of E. coli (DH5alfa) cells, using the Qiagen Tip-100 purification method according to the suppliers (Qiagen GmbH, Germany) instruction. The presence of the substitutions in all mutant B-segment plasmids were confirmed by nucleotide sequence analysis. Secondary structure

prediction of the altered 3'-UTR region of the coding-strand B-segment RNA was performed using the Mfold program (version 3.0) (Zuker, 1989).

Co-transfection of fowlpox T7 infected QM5 cells with the IBDV cDNA plasmids.

Purified pMB plasmids were co-transfected with the pHB-36W plasmid into QM5 cells. Plasmid pHB-36W contains the A-segment cDNA of CEF94 preceded by a T7 polymerase promoter, and followed by the Hepatitis Delta Virus ribozyme (Boot et al., 1999). Prior to co-transfection, the QM5 cells were infected for 1 hour with fowlpox T7 virus (Britton et al., 1996) as described (Boot et al., 1999). After transfection the cells were rinsed with PBS, covered with complete medium and incubated for 24h at 37° C (5.0% CO_2). Subsequently, the cells and supernatant were freeze-thawed once, filtered through a 200-nm-pore-size filter (Acrodisc, Gelman Sciences) and stored at –20° C. All transfection experiments were performed at least 4 times independently. The amount of infectious rIBDV particles was determined by a $TCID_{50}$ (50% Tissue Culture Infectious Dose) assay. This analysis was performed by infecting fresh QM5 cells in a 96-wells plate with 10-fold dilutions of the filtered transfection lysates. rIBDV infected wells were visualized in an Immuno Peroxidase Monolayer Assay (IPMA) using an IBDV specific Mab directed against VP3 (Boot et al., 1999). The specific staining of IBDV infected cells using specific antibodies is nessecarry, as some of the rescue mutant (i.e. vMB-10), resulted only in individually infected cells which did not yield plaques or CPE after 48h of incubation.

Serial passages of rescued rIBDV. To determine the stability of the introduced mutations we serially passaged the mutant rCEF94 for 4 times on QM5 cells. During each passage the freeze-thawed lysate of a culture was filtered through a 200-nm-pore-size filter (Acrodisc, Gelman Sciences), and 10% of the volume was used to re-infect a fresh culture of QM5 cells.

Sequence determination of the 3'-UTR B-segment of rIBDV. To determine the 3' termini of the plus strands of the B-segments of the vMB-1R2R of either the 4th passage on QM5 cells, or from bursa derived virus, we purified genomic RNA by differential centrifugation and Proteinase K treatment as described before (Boot et al., 1999). Genomic dsRNA was desolved in 10 µl polyA buffer (50 mM Tris-HCL (pH 7.9), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 250 mM NaCl, 1 mM DTT, 50 µg/ml BSA, 0.04 units

RNasin (Promega), in DEPC treated water) with 0.25 mM ATP, and 1 unit polyA polymerase (Pharmacia). After incubation at 37°C for 30 min the enzyme was denaturated by incubation at 75°C for 10 min. The polyadenylated dsRNA was purified by phenol/chloroform extraction (two times) and the purified RNA was precipitated by ethanol/NaAc. The dsRNA was dissolved in 25 µl DEPC-treated water. For the generation of single stranded copy DNA (cDNA) 10 µl of purified polyadenylated RNA was used and 4 µl AmpliTaq buffer II (10x, Perkin-Elmer), 4 µl $MgCl_2$ (25 mM, Perkin-Elmer), 4 µl DTT (0.1 M, Gibco), 2 µl dNTPs (10 mM each) and 4 µl UAPdT primer (2 pmol/µl, Table 4) were added. After incubation at 70°C for 2 min this mixture was immediately transferred onto ice. The dsRNA-primer mixture was subsequently incubated at 52°C for 2 min, after which 1 µl of reverse transcriptase (Superscript II, Gibco/BRL) was added, or no reverse transcriptase (negative control) and incubation at 52°C was continued for 1 h. The reverse transcription reaction was terminated by incubation at 75°C for 10 min. The genomic dsRNA and the RNA of the RNA/DNA hybrid were destroyed by addition of 0.5 µl mixture of RNase A (20 mg/ml, Calbiochem) and 0.5 µl RNase H (1.2 U/µl, Pharmacia) and incubation at 37°C for 20 min. To amplify the single stranded cDNA we used a nested PCR. For the first PCR we used the primers UAP (5'GGCCACGCGT CGACTAG3') and BC9 (5'GCTCTAGAAC TTGTGGAAAC AAGCGA3'), for the nested PCR we used the primers UAPN (5'CCACGCGTCG ACTAGTAC3') and BC10 (5'GCTCTAGATC AAGAACCCAC AGACCG3'). The following conditions were performed for the first PCR: 2 µl cDNA, 10 pmol of each specific primer, 2.5 mM $MgCl_2$, 1*AmpliTaq buffer II (Perkin-Elmer), 50 mM each dNTP and 1 unit of AmpliTaq polymerase (Perkin-Elmer), in a total volume of 50 µl. After incubation at 94°C for 1 min, the amplification was performed in 30 cycles through the different temperature levels: 94°C for 15 s, 52°C for 15 s, and 72°C for 60 s. For the nested PCR, 15 µl of the first PCR amplification reaction was separated in an agarose gel (1.5%) and the specific product (372 bp) was stinged 3 times with a needle and mixed with a 50 µl complete nested-PCR mixture. The conditions of the nested PCR were the same as in the first PCR with the following adaptations: a hybridization temperature of 54°C and 3.5 mM $MgCl_2$. The resulting nested PCR product (309 bp) was gel purified and sequence analysis was directly performed on the PCR products (Table 4).

Virulence of rescued IBDV in SPF chickens. Rescued IBDV containing the A-segment of the very virulent IBDV strain D6948 (pDA-60), in combination with the B-segment of D6948 (pHB-55, yielding rD6948), or the B-segment of Cef94 (pHB-52, yielding vDACB) or with the modified B-segment of CEF94 (pMB-1R2R, yielding vDACB-1R2R) was first propagated on embryonated eggs by inoculating supernatant of a transfection experiment into 11-days old embryonated eggs via the chorioallantoic membrane route. After five days of incubation the embryos (dead or alive) were recovered, homogenized in a Sorval Omni-mixer (3 times 10 sec, max. speed), clarified by centrifugation (6000 g, 10 min.), and subsequently stored in aliquots at –70° C. The virus titer (50% embryo lethal dose, ELD_{50}) in these samples was determined using 11-days old embryonated eggs. Four groups of 35 chickens (7-days old) were housed separately in isolators and received orally 50 ELD_{50} rIBDV in PBS, or only PBS. (negative control group). At 7, 14 and 21 days post infection 5 animals were removed aselected, and a blood sample was taken before euthanazation. Bursa and body weights were determined of all chicks euthanized, Samples from the bursa of Fabricius taken at necropsy were fixed in 10% neutral-buffered formalin, dehydrated, embedded in paraffin wax, sectioned and stained with Hematoxylin-Eosin (H & E). The histopathologic bursal lesion score (HBLS) was determined by microscopic analysis of the bursa following the using the European Pharamacopoeia classification: 0 = absence of damage; 1 = necrosis of isolated follicles; 2 = moderate general depletion of lymphocytes or severe depletion limited to a few follicles; 3 = severe depletion of lymphocytes in more than 50 per cent of follicles; 4 = remains of follicular contours showing a few lymphocytes with hyperplasia of related tissues, cysts, thickened and folded epithelium; 5 = loss of the entire follicular structure with associated fibroblasts.

Virus neutralisation assay. Blood serum samples of chickens were used to determine the amount of IBDV neutralizing antibodies. Diluted sera were incubated with 30 - 300 $TCID_{50}$ of IBDV strain CEF94 at 38°C and 5% CO_2 for 1 hour. After incubation, the mixture was transferred to monolayers of QM5-cells in flat-bottom 96-wells plates (Greiner, Frickenhausen, Germany) and incubated for 24 hours at 38°C and 5% CO_2. Thereafter the monolayers were washed with PBS, dried overnight and fixed in 4% paraformaldehyde for 10 minutes at 4°C. Infective centers were detected in an IPMA using VP3 specific antibodies (see above).

Results

The 3'-terminal ends of the IBDV mRNAs does not contain a polyadenylated tail, but is in stead predicted to fold into a small stem-loop structure comprising nt -20 to -2 in case of the A-segment, and nt -23 to -3 in case of the B-segment (Fig. 6). To study the function of this stem-loop structure, and to analyze the influence of an altered stem-loop structure on viral viability and replication efficiency, we have mutated several nucleotides which are involved in formation of the stem-loop structure of the B-segment. The desired mutations were introduced by PCR amplification of only a small part of the 3'-part of the B-segment cDNA sequence. This mutated 3'-region was subsequently transferred to the full-length B-segment plasmid clone of CEF89 (pHB-52), and verified by sequence analysis. The mutant B-segments cDNA plasmid was co-transfected with the A-segment cDNA plasmid of CEF89 (pHB-36W) into QM5 cell which were infected with recombinant fowlpox virus expressing T7 polymerase prior to transfection, and the presence and phenotype of infectious rescued virus was determined either in vitro or in vivo.

Stabilizing mutations in the middle of B-segment stem-loop. Both the A- and B-segment have in the middle of the 3'-UTR stem-loop structure one (A-segment) or two (B-segment) U-G basepairs (Boot et al., 1999). Although U-G basepairs are tolerated, they do not contribute to the stability of a stem-loop structure. To determine whether a U-G basepair is an essential part of the B-segment stem-loop structure we have mutated either the upper U-G (pMB-1) or the lower U-G (pMB-2), by exchanging the uracil for an cytosine (Fig. 6). The plasmids were separately co-transfected in 4-fold with the CEF94 A-segment plasmid into QM5 cells and the viral titer 24h post transfection we determined. We could easily rescue infectious IBDV virus from these two mutated B-segment plasmids, although the rescue efficiency ($TCID_{50}$ at 24h after transfection) was about 10 (pMB1) or 100-fold (pMB-2) lower in comparison to cDNA encoding the wild-type B-segment stem-loop (Table 4). The rescued virus was subsequently serial passed for 4 times, and a single step growth curve for each of them was performed (data not shown). No difference was found in the replication behavior of these two viruses in comparison to the wild-type CEF94, indicating that

the introduced mutations likely have already been lost during the 4 serial passages. Despite the equal growth characteristics, it appeared from sequence analysis of the 4th passage mutant viruses (vMB-1 and -2) that the introduced mutations were in all cases still present, but additional mutations had been acquired (Table 5). To evaluated whether the readily acquired mutations were stable during prolonged cultivation, we continued the serial passages for an additional 6 times (p10). No additional mutations in the B-segment of the 3'-UTR were found in p10 in comparison to p4 (data not shown).

A modified stem-loop structure. On basis of the reversion mutation we assumed that not the primary sequence of the stem-loop structure is very important, but rather the composition, secondary structure and its stability (predicted energy value). To gain prove for this assumption we designed a stem-loop structure on basis of the sequences we found in p4 culture of vMB-1 and –2 (see Fig. 7). The pMB-1R2R stem-loop contains 4 mutations in comparison to the wild-type situation, yielding a stem-loop structure in which 4 of the 6 internal basepairs of stem have been altered, while the nucleotide composition, predicted secondary structure, stability, and loop sequence has been preserved. Rescue of this virus was as efficient as wild-type virus (Table 4), already indicating that this artificial stem-loop structure could replace the wild-type stem-loop structure without (partially) loss of function. Sequence analysis after the 4th serial passage indeed showed that this artificial stem-loop structure was completely preserved (see Table 5).

Very virulent IBDV with a modified B-segment 3'-UTR stem-loop structure. All the stem-loop mutations have been introduced into the genome of the cell culture adapted isolate CEF94. The specific mutations for growth on cell culture are located in the VP2 region of the polyprotein, which encoded by the A-segment. As a result of the adaptation mutations, the cell culture adapted isolates are no longer virulent for young chicken, so no data on (loss of) virulence due to introduced stem-loop mutations can be obtained using this genomic back-ground. To analyze the influence of the modified stem-loop structure on the virulence of very virulent IBDV we rescued a segment reassortent virus, in which the A-segment (pDA-60) of a very virulent D6948 strain is combined with the wild-type (pHB-52) or the modified stem-loop containing

B-segment of CEF94 (pMB-1R2R). Plasmids were co-transfected into QM5 cells, which support the initial replication. The lysate of the transfected cells is, after filtration, inoculated into embryonated eggs, which support the replication of very virulent IBDV. After egg amplification and titration, groups of 35 SFP-chickens were orally infected with 50 ELD_{50} of each rescued virus at the age of 7 days . At 7 , 14 and 21 days post infection 5 birds of each group were removed aselect, and virus neutralizing titers, bursa body weight ratio's and the damage of the bursa of Fabricius was determined (Table 6 and 4). No differences were found between any of these parameters after infection with either wild-type very virulent (rD6948), the virus containing the modified stem-loop structure in the CEF94 B-segment (vMB-1R2R), or the segment reassorted virus containing an un-modified 3'-UTR B-segment stem-loop structure (vDACB).

Discussion

Birnaviruses differ in their genome replication from all other studied viruses as the RNA dependent RNA polymerase (VP1) is also acting as the Viral Protein genome-linked (VPg) molecule. Limited knowledge is present about steps in the replication and translation and replication of birnaviruses genomes. The birnavirus mRNA is lacking a 5'-Cap structure, and only possesses a small 5'-UTR (less then 100 nt). The small 5'-UTR prohibits the formation of an extensive secondary and tertiary structure found to act as Internal Ribosome Entry Sites (IRES) in other RNA viruses lacking a 5'-Cap structure (e.g. picorna- and flaviviruses). Furthermore the mRNA of birnaviruses are lacking a 3'-poly-A tail. To get insight into the replication and translation strategies used by birnaviruses we have mutated the predicted stem-loop structure of the B-segment of IBDV, using site directed mutagenesis. Rescue of infectious virus from the cDNA replacing the U-G basepair with a C-G basepair in the 3'-UTR stem-loop strucutre of the B-segment was impaired. However, once infectious virus was obtained it had wild-type-like replication kinetics. This suggests that either the introduced mutations were readily lost (reversion) during the first rounds of replication, or that second site mutation were acquired which compensated the introduced mutations. Sequence analysis of rescue virus after the 4th and 10th passage indeed revealed both no reversion had occurred, but that indeed second site mutations had been acquired (Table 4). The second site mutations found in these viable mutants were all present in the stem-loop structure, strongly supporting the formation of this stem-loop in the nature course of infection, and a functional role for this secondary structure in the viral life cycle. The generation of a modified stem-loop structure in which 4 of the 6 internal basepairs were changed yielded virus (vMB-1R2R) with the same efficiency after transfection, and this virus had fully conserved the introduced mutations after 4 serial passages. The generation of a segment reassorted virus containing the A-segment of the very virulent D6948 isolate, and a CEF94 derived B-segment with the modified 3'-UTR stem-loop structure (vDACB-MB1R2R) showed equal virulence parameters, when analyzed in 7-days-old SPF layer-type of chickens. Sequence analysis of the virus recovered form the infected bursa showed that the mutations had also been maintained during the infection in the natural host. Using rationally designed or randomly generated 3'-UTR stem-loop structures in either the A-segment, B-segment, or both segment at the same time, it

is also possible to rescue a modified IBDV which shows an attenuated phenotype and which can be used as efficacious live vaccine. As shown above a modified stem-loop structure is maintained both in vitro and in vivo, showing that such a modified IBDV is also safe.

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Legends to the Figures:

Fig. 1: Schematic representation of the plus-strand of the dsRNA genome of IBDV. The open reading frames of the viral proteins are represented by boxes, while the Viral Protein genome-linked (VPg) is represented by a circle. The predicted stem-loop structure at the 3'UTR of both the A and B-segment (see Fig. 2) are also indicated.

Fig. 2: Prediction of the secondary structure of the 3'-terminus of the coding strand of the A- and B-segments of IBDV using version 3.0 of the Mfold program. Both 3'-termini are predicted to fold in a stable stem-loop structure. The last nucleotides (2 cytosines in case o the A-segment and 3 cytosines in case of the B-segment) are not involved in the stem formation. The predicted stability of the stem-loop structure is given in kcal/mole below th sequence.

Fig. 3: Prediction (Mfold program version 3.0) of the secondary structure of the 3'-termina nucleotides of the coding strand the mutant B-segments. Substituted nucleotides are indicated by arrows and are given in bold face. The predicted stability of the mutant sequence is given in kcal/mole.

Fig. 4: Wild-type and mutant rCEF94 were used to inoculate a near confluent monolayer (QM5 cells with an MOI of 1 to 10, and incubated at 37°C for 50 hours. At regular timepoin after inoculation, we removed 0.5 ml of the supernatant and the amount of infectious IBD' particles ($TCID_{50}$) was determined for each sample. Wild-type rCEF94 virus was inoculate both with an MOI of 1 (rCEF94 10^6) and with an MOI of 10 (rCEF94 10^7).

Fig. 5: Untranslated genomic nucleic acid sequences of different serotype I IBDV strains.

Fig. 6: The uracils encompassed by the wild-type stem-loop structure of the B-segment IBDV were replaced by a cytosine in MB1 (uracil at position –18) and MB2 (uracil at position –19).

Fig. 7: Four nucleotides which are all involved in the stem-loop formation of the 5"-UTR of the coding strand of the IBDV B-segment were mutated. The predicted stem-loop encoded by this mutant plasmid (pMB-1R2R) differs from the wild-type stem-loop structure (pHB-52) in respect to the sequence of the base pairs in the internal part of the stem. The overall Sequence composition is conserved, just as the numbers of G-C and G-U basepairs in the stem. The predicted stability of the modified stem-loop structure (-11.4 kcal.mol) is only slightly different from the predicted stability of the wild-type stem-loop structure (-11.7 kcal.mol).

Table 1: Analysis of the rD6948^{HT}-D23 IBDV strain

Virus	A-segment cDNA plasmid	B-segment cDNA plasmid	Rescue efficiency*	Cyto-Pathogenic Effect		
				24h	48h	72h
rD6948^{HT}-BC	pJH60-HT	pHB-34Z	5.3 (0.4)	+	+++	+++
rD6948^{HT}-D23-BC	pJH60-HT-D23	pHB-34Z	2.4 (0.4)	-	+	++

* The mean titer of the rescued IBDV (50% Tissue Culture Infectious Dosis ($TCID_{50}$)) of four independent transfection experiments is given. The standard deviation is given between brackets

Table 2: Amount of infectious IBDV particles (10Log $TCID_{50}$) 24h after co-transfection of mutant B-segment with unmodified A-segment cDNA plamids

Virus	NBK/pp	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Average
Neg.	818/149	0.0	0.0	0.0	0.0	0.0
rCEF94	818/148	4.8	4.8	4.7	4.7	4.8
rCEF94-MB1	824/91	3.5	3.5	3.7	3.5	3.6
rCEF94-MB2	824/91	3.0	2.7	2.6	1.5	2.5
rCEF94-MB3	824/91	2.0	2.3	2.3	2.3	2.3
rCEF94-MB4	824/82	0.3	0.0	0.5	0.3	0.3
rCEF94-MB5	824/82	0.3	0.7	1.0	0.5	0.8
rCEF94-MB6	824/82	2.2	1.4	2.0	1.6	1.8
rCEF94-MB7	818/149	1.3	1.5	1.5	1.5	1.5
rCEF94-MB8	818/150	0.3	0.3	0.0	0.0	0.2
rCEF94-MB9	818/150	1.3	1.5	1.3	0.3	1.1
rCEF94-MB10	824/82	0.7	0.5	0.8	0.5	0.8
rCEF94-MB11	824/91	0.0	0.0	0.0	0.0	0.0
rCEF94-MB12	824/91	0.0	0.0	0.0	0.0	0.0

Table 3: Nucleotide sequences of the B-segment cDNA plasmids, and the resulting sequence of the 4th passage of the corresponding rescued IBDV.

Name	cDNA plasmid derived sequence[1]	Energy[2]	Sequence in virus of the 4th passage[3]	Energy[2]
rCEF94	UCCCCGGCCUUCGCCUGCGGGGGCCCCC	-11.7	UCCCCGGCCUUCGCCUGCGGGGGCCCCC	-11.7
rCEF94-MB-1	---------C----------	-14.2	---------C----------U----	-11.4
rCEF94-MB-2	----------C----------	-14.2	--------U-C----------	-11.7
rCEF94-MB-3	---------CC----------	-16.7	---------GC----------	-10.1
rCEF94-MB-4	----------C----------	-10.4	----------A-C------U--------	-11.5
			----------C------U--------	-7.8
rCEF94-MB-5	----------GC----------	-9.2	---------G-**----------U----	-6.1
			---------G-**----------	-8.0
rCEF94-MB-6	----------GC----GC---------	-13.3	----------GC---AGC---------	-13.3

rCEF94-MB-7	----------C-----	-8.5	----------*-----	-11.7
rCEF94-MB-8	----------CG----	-7.8	----------**----	-11.7
rCEF94-MB-9	------CG----------CG----	-11.3	------CG-C----------UG----	-11.7
			------CG----------UG----	-8.6
rCEF94-MB-10	----G----------	-14.6	ND	
rCEF94-MB-11	----G----------G--	-12.3	NV	
rCEF94-MB-12	---GG----------	-16.7	NV	
rCEF94-MB-13	--GGG----------	-19.6	NV	

1) The given sequences represents nucleotide 2799 to 2827 of the positive strand of the B-segment of IBDV as found for wild-type virus (CEF94, GenBank number AF194429). The nucleotides which are identical to the wild-type sequence are represented by a dash in the mutant viruses (rCEF94-MB1, -12), while the mutations which are encoded by the generated mutant cDNA plasmids are given as letters.

2) The energy value resulting from the Mfold (Version 3.0) secondary structure prediction is given in (kcal/mole).

3) The given sequences represents nucleotide 2799 to 2827 of the positive strand of the B-segment of IBDV as found for wild-type virus, or as found in the 4th passage after rescue from the mutant B-segment cDNA plasmids. Mutations which differ according to the corresponding cDNA sequences are given in bold; Reversion ot the wild-type sequences are indicated by an asterisks. ND = Not Done; NV = Not Viable.

Table 4

Virus	Mutation	Co-transfected cDNA plasmids		Recsue[a]	$TCID_{50}$[b]
		B-segment	A-segment		
Mock	Neg. control	pHB-52	-	0/16	-
rCEF94	Pos. control	pHB-52	pHB-36W	16/16	4,6 (0.6)
vMB-1	$U^{-18} \wedge C$	pMB-1	pHB-36W	4/4	3,6 (0.1)
vMB-2	$U^{-19} \wedge C$	pMB-2	pHB-36W	4/4	2,5 (0.7)
vMB-1R2R	$C^{-5} \wedge U$ & $U^{-18}U^{-19}C^{-20} \wedge CCU$	pMB-1R2R	pHB-36W	3/3	4,5 (0,3)
rD6948	Pos. control	pHB-55	pDA-60	nr[c]	nr[c]
vDACB	Pos. control	pHB-52	pDA-60	nr[c]	nr[c]
vDACB-1R2R	$C^{-5} \wedge U$ & $U^{-18}U^{-19}C^{-20} \wedge CCU$	pMB-1R2R	pDA-60	nr[c]	nr[c]

a) Number of times that infections virus obtained after transfection / number of transfection experiments
b) Amount of infectious particles at 24h post transfection is given as 50% Tissue Culture Infectious Dosis as Log_{10} (standard deviation)
c) nr: not relevant, as viruses containing an A-segment of the very virulent D6948 strain are unable to grow in tissue culture

Table 5: Nucleotide sequences of the B-segment cDNA plasmids, and the rescued viruses form these plasmids after serial passage

Name	cDNA plasmid derived sequence[1]	Energy[2]	Sequence in virus of the 4th passage on QM5 cells[3]	Energy[2]	Sequence in virus after 1 passage in SPF chickens[3]	Energy[2]
rCEF94	UCCCCGGCCUUCGCCUG CGGGGGCCCCC	-11.7	UCCCCGGCCUUCGCCUG CGGGGGCCCCC	-11.7	ND[4]	
vMB-1	---------C----------	-14.2	---------C----------**U**----	-11.4	ND[4]	
vMB-2	----------C----------	-14.2	--------**U**-C----------	-11.7	ND[4]	
vMB-1R2R	--------UCC----------U----	-11.4	--------UCC----------U----	-11.4	ND[4]	
vDACB-1R2R	--------UCC----------U----	-11.4	NR[3]		--------UCC----------U----	-11.4

1) The given sequences represents nucleotide 2799 to 2827 of the positive strand of the B-segment of IBDV as found for wild-type CEF94 (GenBank accession number : AF194429). The nucleotides which are identical to the wild-type sequence are represented by a dash in the modified viruses, while the mutations which are encoded by the generated mutant cDNA plasmids are given in plain letters, while acquired mutations after transfection and during passaging are given in bold letters.
2) The energy value resulting from the Mfold secondary structure prediction using the sequence as shown in the preceding column is given in (kcal/mole).
3) NR = Not Relevant, as this virus is unable to grow on QM5 cells
4) ND = Not Done

Table 6: Post mortem analysis of chickens infected with 50 ELD_{50}, and euthanized at 7, 14 or 21 days post infection

Virus	Mean Weight[1] (g)			Mean BBW Ratio[1] x1000		
	7 days PI	*14 days PI*	*21 days PI*	*7 days PI*	*14 days PI*	*21 days PI*
PBS	132 (7)	228 (11)	343 (35)	3.9 (1.5)	6.7 (2.1)	6.2 (1.4)
D6948	120 (8)	212 (35)	294 (16)	1.0 (0.3)	1.1 (0.2)	0.9 (0.2)
vDACB	97 (9)	187 (17)	298 (57)	1.5 (1.0)	1.0 (0.2)	0.9 (0.2)
vDACB-1R2R	108 (5)	195 (21)	286 (14)	1.7 (0.4)	1.2 (0.3)	1.1 (0.2)

1) The given values are the mean of the values of 5 different chickens (Standard Deviation).

Table 7: Antibody levels and Post mortem analysis of chickens infected with 50 ELD50

Virus	VN-titers[1,2]			HBLS[1,3]		
	7 days PI	*14 days PI*	*21 days PI*	*7 days PI*	*14 days PI*	*21 days PI*
Mock	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
D6948	7.6 (1.1)	10.8 (1.1)	12.0 (0.0)	5.0 (0.0)	4.8 (0.4)	4.6 (0.5)
vDACB	7.4 (1.3)	11.8 (0.5)	11.6 (0.6)	5.0 (0.0)	5,0 (0,0)	4,4 (0,5)
vDACB-1R2R	8.4 (1.5)	11.0 (1.0)	11.4 (0.9)	5.0 (0.0)	4.8 (0.5)	4.2 (0.4)

1) The given values are the mean of the values of 5 different chickens (Standard Deviation).

2) Virus neutralizing titers are given as Log_2 values. Maximum value which can be scored in the used VN test is $12Log_2$. Chickens are considered to be protected is they have a VN-titer of $7Log_2$ or higher after immunisation.

3) Histopathologic Bursa Lesion Score (HBLS) has been determined using the following scale: 0 = absence of damage; 1 = necrosis of isolated follicles; 2 = moderate general depletion of lymphocytes or severe depletion limited to a few follicles; 3 = severe depletion of lymphocytes in more than 50 per cent of follicles; 4 = remains of follicular contours showing a few lymphocytes with hyperplasia of related tissues, cysts, thickened and folded epithelium; 5 = loss of the entire follicular structure with associated fibroblasts.

Claims

1. An isolated or recombinant Birna virus provided with an alteration of the primary sequence of an essentially untranslated region of its genomic RNA.

2. A virus according to claim 1 wherein said region comprises a 3'untranslated region its genomic RNA.

3. A virus according to claim 2 wherein said alteration provides a secondary stem-loop structure of said region with a predicted stability within a range of -15 kcal/mol. to -8 kcal/mol.

4. A virus according to anyone of claims 1 to 3 wherein said alteration comprises a nucleotide substitution or deletion.

5. A infectious bursal disease virus according to anyone of claims 1 to 4.

6. 7. A virus according to anyone of claims 1 to 5 provided with an alteration of th primary sequence of an essentially untranslated region of its segment B of its genomic RN whereby an essentially untranslated region of its segment A comprises a sequence characteristic for wild-type virus.

7. A virus according to anyone of claims 1 to 5 provided with an alteration of the primary sequence of an essentially untranslated region of its segment A of its genomic RN whereby an essentially untranslated region of its segment B comprises a sequence characteristic for wild-type virus.

8. Use of a virus according to anyone of claims 1 to 7 for the production of a vaccine.

9. Use according to claim 8 for the production of a vaccine for the treatment or prevention of IBDV infections.

10. A vaccine comprising a virus according to anyone of claims 1 to 7.

11. A vaccine according to claim 11 which is a modified live vaccine.

12. A method for the preparation of an attenuated Birna virus comprising providing an alteration of the primary sequence of an untranslated region of its genomic RNA

13. A method according to claim 12 wherein said alteration comprises a nucleotide substitution or deletion.

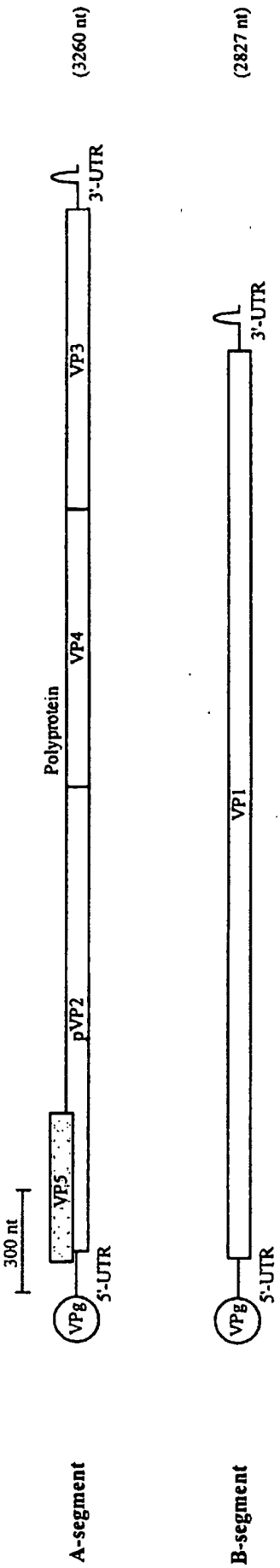
14. A method according to claim 12 or 13 wherein said alteration provides a secondary stem-loop structure with a predicted stability within a range of -15 kcal/mol. to -8 kcal/mol

15. A method according to claim 14 wherein said stability is within a range of -13 kcal/mol. to -10 kcal/mol.

16. A virus obtainable by a method according to anyone of claims 12 to 15.

17. A vaccine comprising a virus according to claim 15.

18. A vaccine according to claim 17 wherein said virus is live.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(2827 nt)
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VP3
VP4
Polyprotein
pVP2
VP5
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5'-UTR
VPg
A-segment
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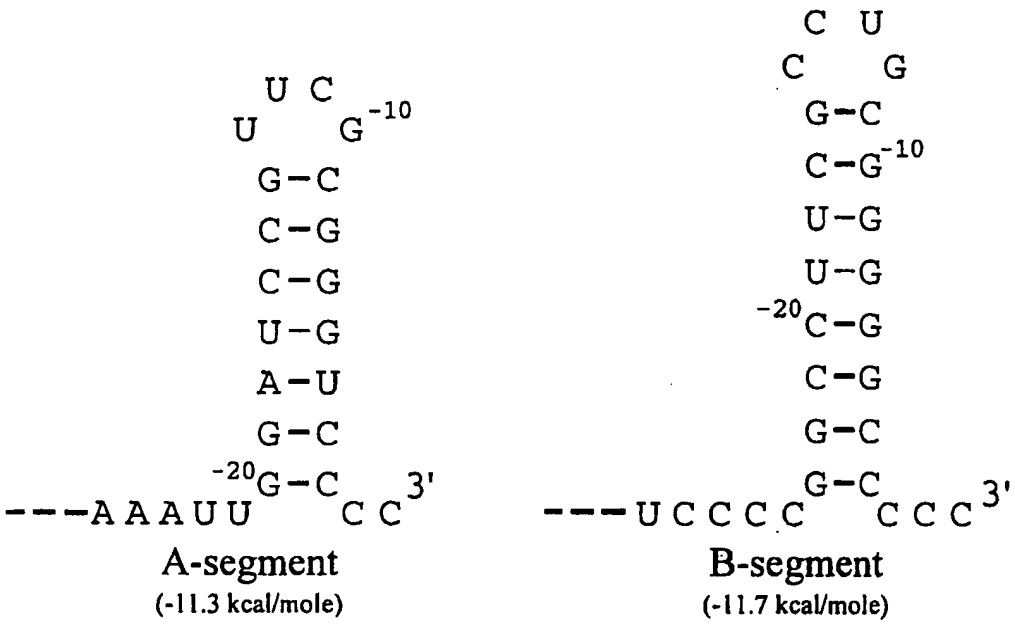
VP1
5'-UTR
VPg
B-segment

Fig. 1

Fig. 2


U C
U G -10
G-C
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C-G
U-G
A-U
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-20 G-C
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A-segment
(-11.3 kcal/mole)
C U
C G
G-C
C-G -10
U-G
U-G
-20 C-G
C-G
G-C
G-C
---UCCCC C C C 3'
B-segment
(-11.7 kcal/mole)

Wild type
B-segment
(-11.7 kcal/mole)
MB1
B-segment
(-14.2 kcal/mole)
MB2
B-segment
(-16.7 kcal/mole)
MB3
B-segment
(-10.4 kcal/mole)
MB4
B-segment
(-9.2 kcal/mole)
MB5
B-segment
(-13.3 kcal/mole)
MB6

Fig. 3

MB7
B-segment
(-8.5 kcal/mole)
MB8
B-segment
(-7.8 kcal/mole)
MB9
B-segment
(-11.3 kcal/mole)
MB10
B-segment
(-14.6 kcal/mole)
MB11
B-segment
(-12.3 kcal/mole)
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MB13
B-segment
(-19.6 kcal/mole)

Fig. 3, contd.

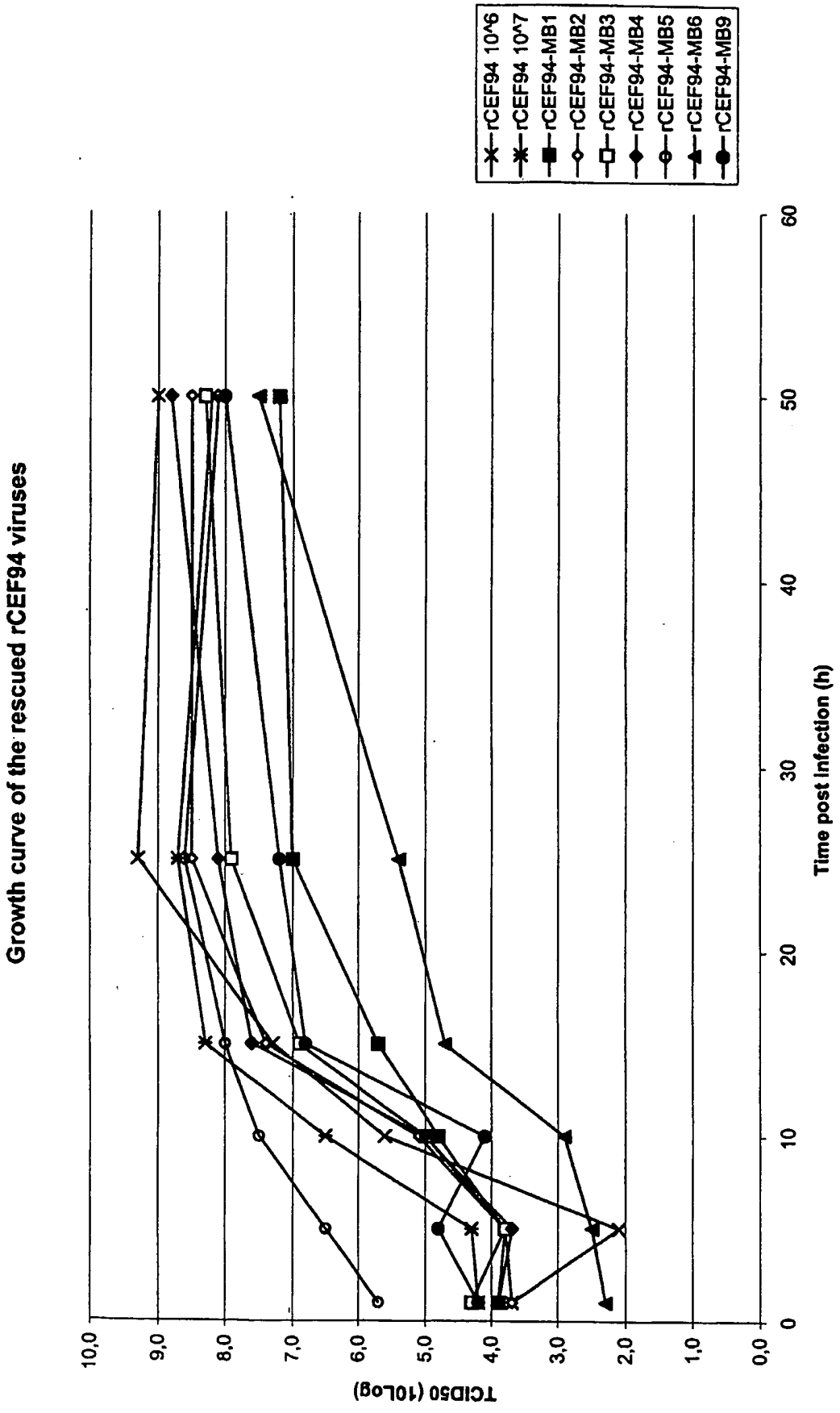

Growth curve of the rescued rCEF94 viruses
rCEF94 10^6
rCEF94 10^7
rCEF94-MB1
rCEF94-MB2
rCEF94-MB3
rCEF94-MB4
rCEF94-MB5
rCEF94-MB6
rCEF94-MB9
TCID50 (10Log)
10,0
9,0
8,0
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
Time post infection (h)
0
10
20
30
40
50
60

Fig. 4

Fig. 5 Untranslated genomic sequences of different Serotype I IBDV strains

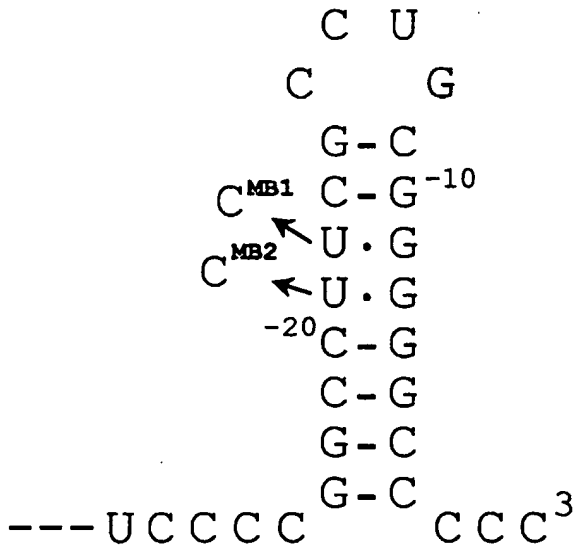
Virus	5'-UTR of the positive strand of A-segments
CEF94	5'GGAUACGAUC GGUCUGACCC CGGGGGAGUC ACCCGGGGAC AGGCCGUCAA GGUCUUGUUC CAGGAUGGAA CUCCUCCUUC UACAACGCUA UCAUUG
D6948	5'---------- ---------- ---------- ---------- ----U----- --C------- ---------- ---------- -----U---- ------
P2	5'---------- ---------- ---------- ---------- ---U-U---- --C------- ---------- ---------- ---------- ------
Cu-1	5'---------- ---------- ---------- ---------- ---U------ --C------- --------G- ---------- ---------- ------
Cu-1M	5'---------- ---------- ---------- ---------- ---------- --C------- --------G- ---------- ---------- ------

Virus	3'-UTR of the positive strand of A-segments
CEF94	G GCUCCUGGGA GUCUCCCGAC ACCACCCGCG CAGGUGUGGA CACCAAUUCG GACUUACAAC AUCCCAAAUU GGAUCCGUUC GCGGGUCCCC3'
D6948	- ---------- ---------- ---------- ----C----- ---------- -C-A------ ---------- ---------- ----------3'
P2	- ---------- ---------- ---------- ---------- ---------- -C-------- ---------- ---------- ----------U3'
Cu-1	- ---------- ---------- ---------- ---------- ---------- -C-------- ---------- ---------- ----------U3'
Cu-1M	- ---------- ---------- ---------- ---------- ---------- -C-------- ---------- ---------- ----------U3'

Virus	5'-UTR of the positive strand of B-segments
CEF94	5'GGAUACGAUG GGUCUGACCC UCUGGGAGUC ACGAAUUAAC GUGGCUACUA GGGGCGAUAA CCGCCGCUGG CUGCCACGUU AGUGGCUCCU CUUCUUGAUG AUUCUGCCAC C
D6948	5'---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
P2	5'---------- ---------- ---------- ---------- ---------- ----U---GC --A-----A- ---------- ---------- ---------- ----A----- -
Cu-1	5'---------- ---------- ---------- ---------- ---------- ---------C ---------- -C-------- ---------- ---------- ---------- -
Cu-1M	

Virus	3'-UTR of the positive strand of B-segments
CEF94	UGGGAACCAC UCAAGAAGAG GACACUAAUC CCAGACCCCG UAUCCCCGGC CUUCGCCUGC GGGGGCCCCC3'
D6948	CCAUGA ---------- ---------- --------C- ---------- ---------- ---------- ----------3'
P2	CAGCCAUGA ---------- ---------- ---------- ---------- ---------- ---------- ----------3'
Cu-1	CAGCCAUGA ---------- ---------- ---------- ---------- ---------- ---------- ----------3'
Cu-1M	CAGCCAUGA ---------- ---------- ---------- ---------- ---------- ---------- ----------3'

Fig. 6



3'UTR B-segment
(-11.7 kcal/mole)

Fig. 1: The uracils encompassed by the wild-type stem-loop structure of the B-segment IBDV were replaced by a cytosine in MB1 (uracil at position –18) and MB2 (uracil at position –19).

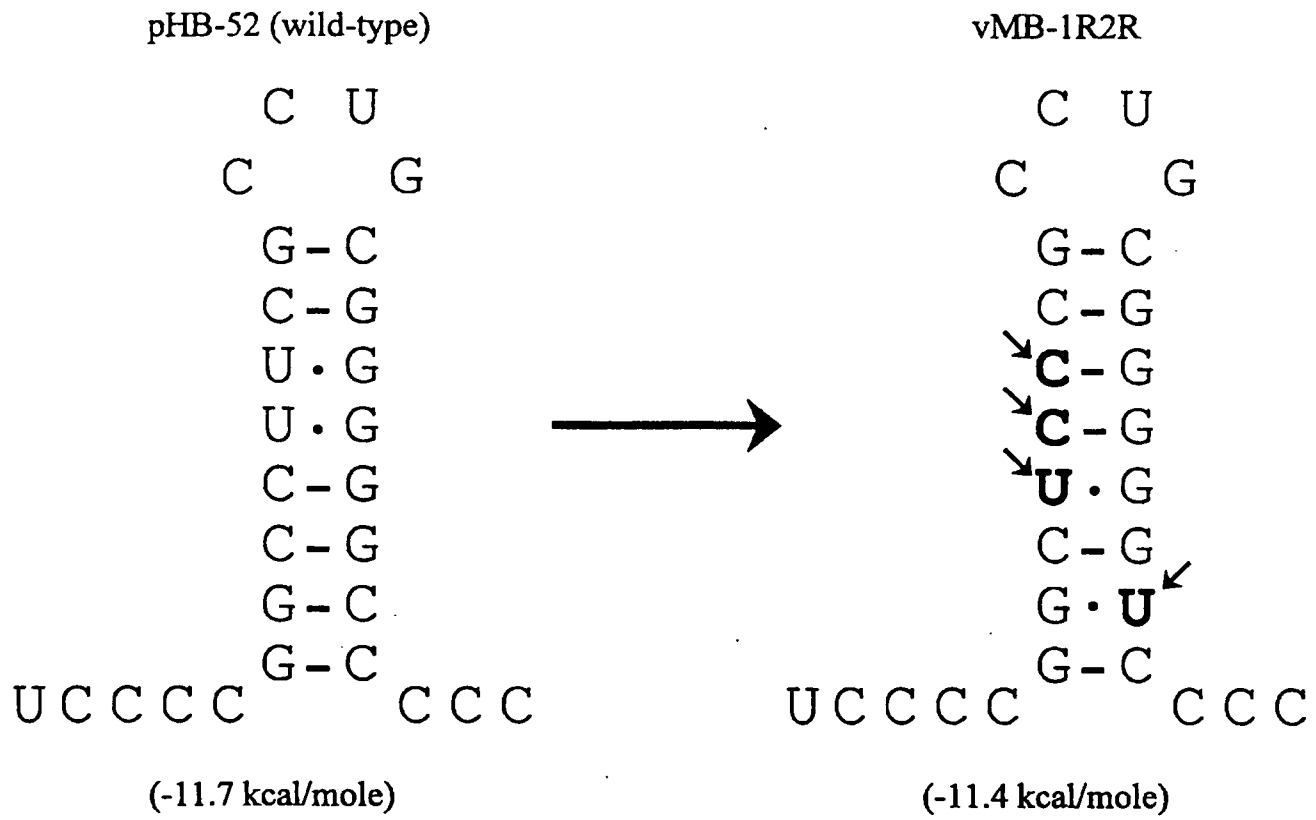


Fig. 1: Four nucleotides which are all involved in the stem-loop formation of the 5'-UTR of the coding strand of the IBDV B-segment were mutated. The predicted stem-loop encoded by this mutant plasmid (pMB-1R2R), differs form the wild-type stem-loop structure (pHB-52), in respect to the sequence of the basepairs in the internal part of the stem. the overall sequence composition is conserved, just as the number of G-C and G-U basepairs in the stem. The predicted stability of the modified stem-loop structure (-11.4 kcal.mole) is only slightly different from the predicted stability of the wild-type stem-loop structure (-11.7 kcal/mole).

Fig. 7